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Journal of Plant Nutrition and Soil Science

DOI:
10.1002/jpln.202000501

Published: 01/12/2021

Publisher's PDF, also known as Version of record

Received: 29 November 2020 | Accepted: 18 September 2021

DOI: 10.1002/jpln.202000501

REGULAR ARTICLE

The short-lived inhibitory effect of *Brachiaria humidicola* on nitrous oxide emissions following sheep urine application in a highly nitrifying soil

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Funding information
Biotechnology and Biological Sciences Research Council, Grant/Award Numbers: BBS/E/C/000I0310, BBS/E/C/000I0320

Abstract

Background: *Brachiaria humidicola* (Bh) has the ability to produce biological nitrification inhibitors (NIs) and release NIs from the root to the soil.

Aims: To compare the effects of growing Bh with *Brachiaria ruziziensis* (Br, which is not able to produce NIs) on soil nitrogen (N) dynamics, N gases and carbon dioxide (CO_2) emissions and nitrifiers and denitrifiers following sheep urine application, a laboratory incubation was conducted in a He/O_2 continuous flow denitrification system (DENIS). This incubation was conducted in the absence of light. Hence the measured effects of Bh and Br on N cycling were the residual effect of biological NIs released into the soil prior to the incubation and released via root death.

Methods: The treatments were: (1) Bh with water application (Bh + W); (2) Bh with sheep urine (Bh + U); (3) Br with water application (Br + W); (4) Br with sheep urine (Br + U).

Results: Results showed that soil NO_3^- concentration increased significantly in the soil with sheep urine application after the incubation. Soil nitrous oxide (N_2O) and nitric oxide (NO) emissions increased immediately after the sheep urine application and peaked twice during the incubation. Cumulative emissions for the first peak were significantly lower from the Bh + U treatment (0.054 kg N ha^{-1}) compared with the Br + U treatment (0.111 kg N ha^{-1}), but no significant differences were observed in the total cumulative N_2O and NO emissions between the Bh + U and Br + U treatment at the end of the incubation. Sheep urine addition did not affect the AOA, *nirS* and *nosZ* gene copies, but significantly increased the AOB gene copies after the incubation.

Conclusions: We conclude that the residual effect of Bh to mitigate N_2O emissions in a highly nitrifying soil is short-lived.

KEYWORDS
Brachiaria humidicola, *Brachiaria ruziziensis*, carbon dioxide, denitrifier, nitrifier, nitrogen gas

1 | INTRODUCTION

Nitrification and denitrification are key processes of the soil nitrogen (N) cycle. Nitrification is a two-step microbially mediated process carried out by chemo-autotrophic nitrifying bacteria, first oxidising ammonium (NH_4^+) to nitrite (NO_2^-) which is further oxidised to nitrate (NO_3^-) (Firestone and Davidson, 1989). During the nitrification and subsequent denitrification, other gaseous forms of N are produced and lost from agricultural soils, such as nitrous oxide (N_2O), nitric oxide (NO) and dinitrogen (N_2). N_2O has been attributed to nitrification, denitrification and nitrifier denitrification processes depending on the soil environmental conditions, such as water-filled pore space (WFPS), O_2 availability, soil pH and temperature (Bateman and Baggs, 2005; Lai et al., 2019; Loick et al., 2016; Wrage et al. 2005). Some studies present NO emitted from soils during nitrification process (Caranto & Lancaster, 2017; Kang et al., 2020; Wang et al., 2016). However, denitrification can also be a major source of NO from soils at high water content and/or under the presence of a carbon (C) source (Ji et al., 2020; Loick et al., 2016; Wu et al., 2017), while N_2 is the final product of denitrification (Knowles, 1982).

Synthetic nitrification inhibitors (NIs) have been widely researched and used to inhibit soil nitrification, for example, dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP) (Chadwick et al., 2018; Chen et al., 2015; Weiske et al., 2001). Following concerns of synthetic NIs passing into human food chains (Anuranga, 2014; Lin et al., 2015; Welten et al., 2016), there has been increasing interests in the role of biological NIs to reduce N_2O emissions and NO_3^- leaching. Some grass species (Florindo et al., 2014; Gopalakrishnan et al., 2009; Subbarao et al., 2008) and crop plants (Huérfano et al., 2016; Subbarao et al., 2013; Sun et al., 2016) have the ability to release compounds from their roots to suppress the nitrifier activity which is termed biological nitrification inhibition (BNI) (Subbarao et al., 2006). *Brachiaria humidicola* (Bh), a typical tropical pasture grass used for grazing livestock, has been reported to release biological NIs from its roots. Active inhibitory compounds have been isolated from the root tissues (e.g., methyl-p-coumarate and methyl ferulate) (Gopalakrishnan et al., 2007), root exudates (e.g., brachialactone) (Subbarao et al., 2009) and shoot tissues (e.g., linoleic acid and linolenic acid) (Subbarao et al., 2008) of Bh.

Previous studies have focused on the effects of pure inhibitory compounds identified from the pasture grass or the root exudates of Bh on soil NH_4^+ transformation and N_2O emissions (Gopalakrishnan et al., 2009; Meena et al., 2014; Subbarao et al., 2008). While experiments have been conducted to explore nitrification inhibition and N_2O emissions from soil planted with *Brachiaria* grasses, including pasture that receive bovine urine deposition (Byrnes et al., 2017; Simon et al., 2020), only a few studies have explored the legacy effects of Bh on N cycling and grain yield of subsequent crops, supplied with N fertiliser, for example, maize (Karwat et al. 2017), and little is known about the residual effect of biological NIs in the rhizosphere after plants like Bh start to die, on N emissions, soil mineral N and soil nitrifiers and denitrifiers.

There is strong evidence that other *Brachiaria* species, for example, *Brachiaria ruziziensis* (Br), are not capable to produce NIs (Fernandes et al., 2011). In this study, Br was selected to compare with Bh (which has the ability to release biological NIs from the roots) to: (1) explore the residual effect of Bh and Br on soil NH_4^+ and NO_3^- concentrations; (2) quantify the N_2O, NO, N_2 and CO_2 emissions in soil sown with these two *Brachiaria* varieties; and (3) determine the residual effect of Bh and Br on soil nitrifiers and denitrifiers. Based on current research, we hypothesised that (1) soil under Bh retains soil NH_4^+, and results in lower NO_3^- concentrations than soil under Br, (2) Bh results in lower N_2O and NO emissions than soil under Br due to the higher BNI capacity of Bh and (3) AOA and/or AOB gene copies may be lower in the soil under Bh treatments than those in the soil under Br treatments.

2 | MATERIALS AND METHODS

2.1 | Soil sampling and physicochemical analysis

A sandy clay loam textured Eutric Cambisol was collected from a typical sheep-grazed grassland in North Wales (53°24'N, 4°02'W). The soil had not been previously grown with Bh and Br. The soil was selected for its known high nitrification rate (Jones et al., 2004) and not necessarily as a typical tropical soil where *Brachiaria* species would be grown. Square intact turves of soil (30 × 30 cm, depth of 10 cm) were collected from three spatially discrete points (at least 10 m apart), which were retained as three replicates. Soil was sieved (2 mm) to remove roots and stones before analysis for a range of chemical properties: 19.4% moisture content (105°C, 24 h), 6.7% organic matter (450°C, 16 h) (Ball, 1964), 2.7% total C and 0.25% total N (CHN2000 Analyzer), pH of 5.9, 1.7 mg N kg^{-1} dry soil as NH_4^+-N (Mulvaney, 1996) and 30.4 mg N kg^{-1} dry soil as NO_3^--N (Miranda et al., 2001).

2.2 | Establishment of BH and BR

To investigate the residual effect of Bh and Br on soil nitrification, greenhouse gas emissions (GHG, N_2O and CO_2), NO and N_2 emissions and nitrifiers and denitrifiers after sheep urine application, two varieties of *Brachiaria* species (Bh and Br) were sown separately in pots containing the field soil. Seeds of Bh and Br were germinated on wetted tissue paper in an incubator (20°C). Then 1.7 kg field fresh soil was added to each pot (diameter: 15 cm; depth: 15 cm) at the same bulk density as the soil at the field site (1.6 g cm^{-3}) (Marsden et al., 2016), and 10 germinated seeds were placed onto the soil surface before covering with 100 g soil. There were 12 pots in total, six pots were grown with Bh and six pots with Br. To stimulate grass growth, the plants were cut to 2 cm above the soil level on day 33 and day 75. At the same time, the equivalent of 25 kg N ha^{-1} as $(NH_4)_2SO_4$ was added to each pot 3 days after each cut to promote the release of the inhibitory compounds (Subbarao, Wang et al., 2007). Note that 50 mL of tap water was added to each pot twice per week to maintain plant growth prior to the incubation experiment. The establishment of Bh and Br was from the beginning of July to the end of November. To stimulate the growth of the tropical grasses, the lights above the plots in the greenhouse were on from October until the end of the cultivation. On day 150 after sowing, the plants and soils were harvested for the incubation experiment (described below).

TABLE 1 Soil characteristics before sheep urine application (day 0) and after the incubation (day 23)

Soil property	Bh + W		Bh + U		Br + W		Br + U	
	Day 0	Day 23	Day 0	Day 23	Day 0	Day 23	Day 0	Day 23
Moisture content (%)	30.3 ± 0.23^{a}	27.7 ± 0.78^{B}	30.6 ± 0.11^{a}	30.1 ± 0.54^{A}	29.4 ± 0.60^{a}	29.4 ± 0.79^{AB}	30.2 ± 0.36^{a}	28.0 ± 0.34^{AB}
Organic matter (%)	6.5 ± 0.15^{a}	6.4 ± 0.06^{AB}	6.4 ± 0.21^{a}	6.6 ± 0.05^{A}	6.3 ± 0.05^{a}	6.3 ± 0.07^{B}	6.3 ± 0.13^{a}	6.5 ± 0.03^{A}
pH	6.6 ± 0.03^{a}	6.0 ± 0.02^{A}	6.6 ± 0.04^{a}	5.3 ± 0.05^{B}	6.3 ± 0.08^{b}	6.0 ± 0.05^{A}	6.5 ± 0.04^{ab}	5.2 ± 0.04^{B}
Electrical conductivity ($\mu S\ cm^{-1}$)	116.8 ± 16.7^{a}	147.8 ± 6.84^{B}	109.3 ± 1.84^{a}	802.3 ± 21.8^{A}	111.0 ± 4.63^{a}	158.3 ± 11.0^{B}	104.5 ± 6.02^{a}	755.3 ± 22.0^{A}
Total carbon ($g\ kg^{-1}$ dry soil)	21.4 ± 0.43^{a}	23.3 ± 0.50^{A}	23.2 ± 1.00^{a}	24.9 ± 1.79^{A}	23.5 ± 0.49^{a}	24.1 ± 0.06^{A}	23.0 ± 0.49^{a}	25.1 ± 0.81^{A}
Total nitrogen ($g\ kg^{-1}$ dry soil)	2.6 ± 0.04^{b}	2.8 ± 0.09^{B}	2.7 ± 0.05^{ab}	3.1 ± 0.04^{AB}	2.8 ± 0.10^{a}	2.8 ± 0.08^{B}	2.7 ± 0.02^{ab}	3.2 ± 0.14^{A}
NH_4^+-N ($mg\ N\ kg^{-1}$ dry soil)	3.3 ± 0.17^{a}	1.3 ± 0.36^{B}	2.7 ± 0.13^{a}	3.2 ± 0.43^{A}	3.1 ± 0.39^{a}	0.15 ± 0.05^{B}	3.3 ± 0.46^{a}	3.6 ± 0.97^{A}
NO_3^--N ($mg\ N\ kg^{-1}$ dry soil)	3.7 ± 0.20^{a}	16.0 ± 2.61^{B}	1.8 ± 0.41^{a}	235.7 ± 15.8^{A}	2.8 ± 0.65^{a}	17.3 ± 3.48^{B}	2.6 ± 0.99^{a}	213.9 ± 9.63^{A}

Values represent means ± SEM. Different letters indicate the significant differences between treatments at day 0 (lower case) and day 23 (upper case) respectively ($n = 3$, $p < 0.05$).

2.3 | Experimental setup

The 23-day incubation experiment was conducted in the Denitrification System (DENIS) at Rothamsted Research (North Wyke) (Cárdenas et al., 2003), using the top (0–7.5 cm) of the intact (12 cm deep) soils including plants (obtained from Section 2.2). The soil cores were placed into 12 stainless vessels (diameter: 14.1 cm) and sealed with stainless steel lids fitted with double 'O' rings. The incubation experiment comprised four treatments with three replicates: (1) Bh with water application (Bh + W); (2) Bh with sheep urine (Bh + U); (3) Br with water application (Br + W); (4) Br with sheep urine (Br + U). The sheep urine used in this experiment had been collected from six Welsh Mountain ewes that had been grazing a permanent pasture at the same site the soil was collected from. The urine had been frozen immediately after collection to avoid N losses during storage. The sheep urine was defrosted the day before application to the soil cores, and the individual urine samples ($n = 6$) were pooled and mixed to generate one urine source (total C, 25.3 g L^{-1}; total N, 11.7 g L^{-1}, NH_4^+-N, 1.09 mg L^{-1}; NO_3^--N, 3.09 mg L^{-1}) of which 670 mg N kg^{-1} dry soil (equivalent to 374 kg N ha^{-1}) was added in the treatments.

The incubation experiment followed a similar approach to previous experiments using this DENIS (Loick et al., 2016; Wu et al., 2017). Briefly, to remove the native N_2 from the soil cores and the headspace, the soil cores were flushed from the base at a flow rate of 30 mL min^{-1} for 48 h using a mixture of He/O_2 (80:20), with the outlet flow from each chamber directed to a number of gas detectors. Once the N_2, N_2O and NO concentrations had reached very low levels, the airflow was decreased to 12 mL min^{-1} to measure the baseline emissions before being switched from the flow through the base to a flow over the soil surface. The sheep urine and water amendments were contained in sealed stainless-steel vessels above the lid of each incubation vessel. In previous protocols, these amendment vessels are usually flushed with He/O_2 (80:20) to remove N_2 (Cárdenas et al., 2003). However, in this experiment, the vessels containing the urine and water were not flushed with He/O_2, to avoid the N losses (via NH_3 volatilisation) from the sheep urine. After the urine and water had attained room temperature, the amendments were applied to the soil by opening the ball-valve connecting the two vessels. At the start of the soil incubation, the soil moisture content was increased to 65% WFPS to optimise conditions for nitrification (Mosier et al. 1996), taking the volume of the urine or water amendments into account. The temperature of the vessels was maintained at 15°C during the flushing phase and the 23-day incubation period after the urine and water applications.

2.4 | Soil sampling and analysis

During the incubation, the system was totally sealed, with all the soil gases displaced initially via mix of He/O_2 (80:20) passed through the soil from below and the outlet flow from each chamber was directed to a number of gas detectors. Thus, fresh soil samples were only collected for analysis before the sheep urine application and at the end of the incubation period. Soil characteristics before sheep urine application and the after the incubation are presented in Table 1. Soil moisture content was measured after oven drying (105°C, 24 h), and the soil organic matter was determined by loss on ignition of dried soil in a muffle furnace (450°C, 16 h) (Ball, 1964). Total soil C and N concentrations were determined on milled oven dried soil samples using a CHN2000 Analyzer (Leco Corp., St. Joseph, MI). Soil pH and electrical conductivity (EC) were measured on fresh soil using standard electrodes [1:2.5 (w/v) soil-to-distilled water]. Extractable NH_4^+-N and NO_3^--N were analysed in the filtrates after extracting 5 g of fresh soil with 25 mL K_2SO_4 (0.5 M) using the colorimetric methods of Mulvaney (1996) and Miranda et al. (2001), and total dissolved C and N were analysed with the Multi N/C 2100 (AnalytikJena, Jena, Germany). Data were expressed on a per kg dry soil basis.

At the same time, 5 g fresh soil from each vessel was collected and stored at −80°C prior to DNA extraction. Soil (0.25 g) was extracted by the the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After extraction, the purity and concentration of extracted soil DNA was determined by the Nanodrop spectrophotometer ND−1000 (Labtech, UK). Polymerase chain reaction (PCR) was carried out on real-time quantitative PCR (QPCR) using the QuantStudioTM 6 flex real-time PCR system (Thermo Fisher Scientific, UK). Three independent QPCR were performed for each gene

TABLE 2 Primer sets used for the real-time PCR

Targeting gene	Primer set	Sequence (5′–3′)	Reference
AOA	Arch-amoAF	STAATGGTCTGGCTTAGACG	Robinson et al. (2014)
	Arch-amoAR	GCGGCCATCCATCTGTATGT	
AOB	amoA–1F	GGGGTTTCTACTGGTGGT	Robinson et al. (2014)
	amoA–2R	CCCCTCKGSAAAGCCTTCTTC	
nirK	FlaCu	ATCATGGTSCTGCCGCG	Zulkarnaen et al. (2019)
	R3Cu	GCCTCGATCAGRTTGTGGTT	
nirS	cd3aF	GTSAACGTSAAGGARACSGG	Zulkarnaen et al. (2019)
	R3cd	GASTTCGGRTGSGTCTTGA	
nosZ	2F	CGCRACGGCAASAAGGTSMSSGT	Zulkarnaen et al. (2019)
	2R	CAKRTGCAKSGCRTGGCAGAA	

and each soil replicate. The 20 μL reaction mixture comprised 10 μL TB Green Premix Ex Taq (TaKaRa, Tokyo, Japan), 0.3 μL of each primer, 0.4 μL ROX Reference dye, 7 μL of sterilised deionised water, and 2 μL template DNA. The primers for quantifying nitrification and denitrification function genes were presented in Table 2. The thermal conditions for the AOA, AOB, *nirK*, *nirS* and *nosZ* were the same as those used in previous studies (Bei et al. 2021; Henry et al. 2006). The standard curves for QPCR were generated by 10-fold serial dilutions of linearised plasmids containing cloned AOA, AOB, *nirK*, *nirS* and *nosZ* genes. The PCR amplification efficiencies of standard curves were 93%–98% with R^2 value of 0.990 to 0.999.

2.5 | Gas sampling and analysis

The airflow from each vessel was automatically directed to a valve that directed the sample to different gas detectors, resulting in one sample being analysed every 8 min from each of the 12 vessels. Thus, one measurement was made every 1.5 h from each vessel. The N_2O and CO_2 concentrations were determined using a gas chromatograph equipped with an electron capture detector (ECD), and a second GC with a helium ionisation detector (HID, VICI AG International, Schenkon, Switzerland) was used to analyse N_2 concentrations. For NO concentrations, a chemiluminescence analyser was used (Sievers NOA280i, GE Instruments, Colorado, USA). The gas flow rate through each vessel was measured daily to calculate the volume of gas required for the flux calculation. The gaseous fluxes were corrected for the surface area and flow rate through the vessels and are presented in the unit of kg N or C ha^{-1} d^{-1}. Cumulative gaseous fluxes were calculated by the area under the curve after linear interpolation between sampling points using the Genstat 19th edition (VSN International Ltd) (Meijide et al., 2010).

2.6 | Statistical analysis

One-way analysis of variance (ANOVA) followed by the LSD test at 5% confidence was used to determine the effect of Bh and Br on soil NH_4^+ and NO_3^- concentrations, cumulative gas emissions (N_2O, NO, N_2 and CO_2) and gene abundance (AOA, AOB, *nirK*, *nirS*, *nosZ*) at the start (day 0) and end (day 23) of the incubation, respectively. All statistical analyses were performed in SPSS Statistics 25.0 (IBM Inc., Armonk, NY).

3 | RESULTS

3.1 | Soil ammonium and nitrate concentrations

At the start of the incubation, there were no significant differences between all the treatments (Bh + W, Bh + U, Br + W, Br + U) for the soil NH_4^+ and NO_3^- concentrations, with average concentrations of 3.1 (ranging from 2.7 to 3.3 mg kg^{-1} soil) and 2.7 (ranging from 1.8 to 3.7 mg kg^{-1} soil) mg kg^{-1} soil, respectively (Table 1). In the Bh + W and Br + W treatments, after the 23-day incubation the NH_4^+ concentration decreased (Bh + W, 3.3 to 1.3 mg kg^{-1} soil; Br + W, 3.1 to 0.15 mg kg^{-1} soil) and NO_3^- increased (Bh + W, 3.7 to 16.0 mg kg^{-1} soil; Br + W, 2.8 to 17.3 mg kg^{-1} soil). Note that 23 days after the sheep urine application, there was a small increase in the NH_4^+ concentration in the urine treatments (Bh + U, from 2.7 to 3.2 mg kg^{-1} soil; Br + U, from 3.3 to 3.6 mg kg^{-1} soil) and a large increase in the NO_3^- concentration in the same treatments (Bh + U, from 1.8 to 235.7 mg kg^{-1} soil; Br + U, from 2.6 to 213.9 mg kg^{-1} soil).

3.2 | Gas emissions

3.2.1 | Nitrous oxide

N_2O emissions increased immediately after the sheep urine application, with maximum fluxes of 0.12 and 0.22 kg N ha^{-1} d^{-1} in the Bh + U and Br + U treatments, respectively (Figure 1a). These fluxes decreased rapidly within the following 23 h and then reached another peak after day 13, with what seem to be broad peaks lasting up to 9 days (day 10 to 19). Fluxes, however, remained high until the end of the incubation.

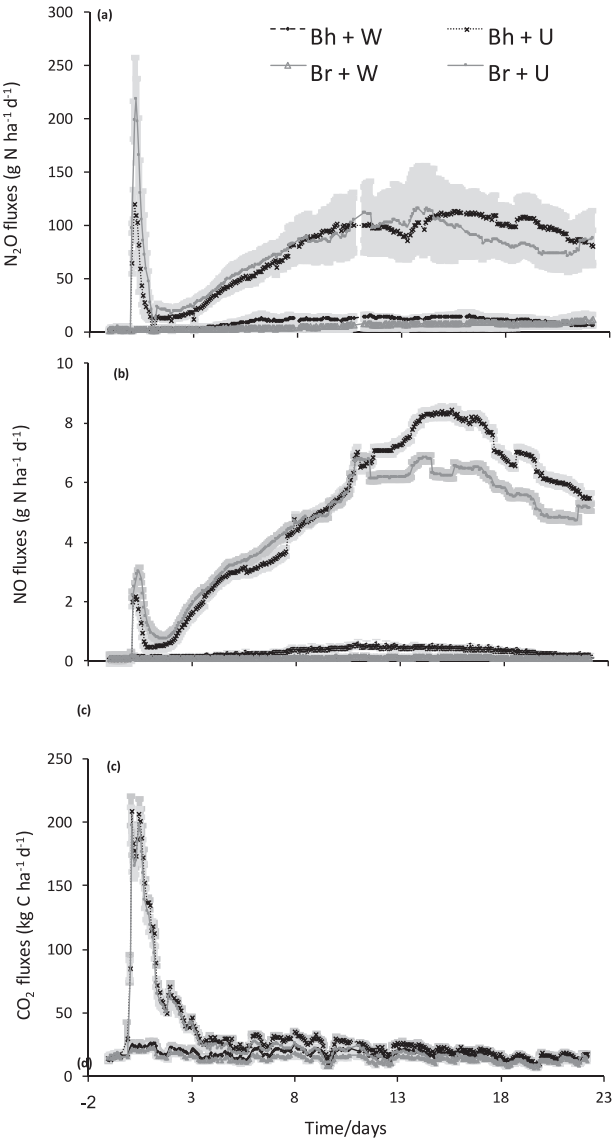

FIGURE 1 Gaseous emissions (average) of N_2O (panel a), NO (panel b) and CO_2 (panel c) during the incubation (urine was applied on the urine treatments at day 0). Error bars represent standard error of the mean ($n = 3$)

N_2O emissions in the Bh + W and Br + W treatments were much lower than that in the treatments with sheep urine application, with average fluxes of 0.009 and 0.006 kg N ha^{-1} d^{-1}, respectively. The cumulative N_2O emission for the first peak in the Br + U treatment (0.11 kg N ha^{-1}) was significantly higher than that in the Bh + U (0.05 kg ha^{-1}) treatment, although no significant differences were observed in the cumulative N_2O emissions for the entire 23 days incubation between the Bh + U and Br + U treatments (Table 3). The cumulative N_2O emissions in the Bh + W and Br + W treatments were significantly lower than that from both urine treatments during both the first peak period and the whole incubation period.

3.2.2 | Nitric oxide

The pattern of NO emissions was similar to the N_2O emissions for all treatments during the 23 days incubation, with the exception that the maximum NO fluxes in the sheep urine application treatments occurred during the second peak on day 14–16 (Figure 1b). The first peak of NO emissions appeared 7.0 and 10.6 h after the urine application in the Bh + U and Br + U treatments, respectively, which was a little later than the peak time of maximum N_2O emissions (3.6 and 5.3 h, respectively) reaching values up to 3 g N ha^{-1} d^{-1}. Cumulative NO emissions in the treatments with the sheep urine application including the two peaks (Bh + U, 0.114 kg N ha^{-1}; Br + U, 0.103 kg N ha^{-1}) were significantly higher than those in the water only treatments (Bh + W, 0.007 kg N ha^{-1}; Br + W, 0.003 kg N ha^{-1}). Nevertheless, no significant differences in NO emissions were observed between the Bh + U and Br + U treatments, or the Bh + W and Br + W treatments during the first peak period or for the whole incubation period. The second NO peak was broader than the initial one (reaching up to ≈ 8 g N ha^{-1} d^{-1}) and had not reached background values at the end of the incubation, but clearly showed fluxes were decreasing from day 16 onwards.

3.2.3 | Nitrogen gas

Fluxes of N_2 were low and decreased continuously from the start of the incubation (data not shown), indicating incomplete flushing of the vessels with contribution of the N_2 that entered the DENIS when non-flushed (He/O_2) urine and water were applied to the soil. Soil-borne N_2 emissions were not observed during the incubation, as expected, as soil moisture conditions were managed to favour nitrification (65% WFPS) (Loick et al. 2021).

3.2.4 | Carbon dioxide

In the Bh + U and Br + U treatments, the CO_2 emissions increased rapidly and peaked at 10.8 h after the urine application (similar to the NO peak in the urine treatments), with the maximum fluxes of 207.2 and 198.9 kg C ha^{-1} d^{-1}, respectively (Figure 1c). The CO_2 emissions decreased afterwards and remained stable (less than ca. 30 kg C ha^{-1} h^{-1}) from day 3.5 to end of the incubation in the Bh + U and Br + U treatments. After the incubation, the cumulative CO_2 emissions in the soil under Br treatments were significantly lower than those in the soil under Bh treatments, following the series: Br + W < Bh + W < Br + U < Bh + U, with the cumulative fluxes of 333.5, 428.5, 654.6, 768.5 kg C ha^{-1}, respectively (Table 3).

3.3 | Nitrifiers and denitrifiers gene copies

At the start of the incubation (day 0), there were no significant differences in the AOA, AOB, *nirK*, *nirS* and *nosZ* gene copies between the

TABLE 3 Cumulative emissions of N_2O and NO (in kg N ha^{-1}) and CO_2 (in kg C ha^{-1}) after 23 days incubation and during the first peak period

Gas	Bh + W	Bh + U	Br + W	Br + U
N_2O (23 d)	0.216 ± 0.026 b	1.73 ± 0.316 a	0.128 ± 0.068 b	1.72 ± 0.324 a
N_2O (first peak)	0.003 ± 0.000 c	0.054 ± 0.010 b	0.004 ± 0.001 c	0.111 ± 0.017 a
NO (23 d)	0.007 ± 0.001 b	0.114 ± 0.009 a	0.003 ± 0.001 b	0.103 ± 0.015 a
NO (first peak)	0.0003 ± 0.0001 b	0.0015 ± 0.0001 ab	0.0003 ± 0.0001 b	0.0025 ± 0.0007 a
CO_2 (23 d)	422.0 ± 10.5 c	761.9 ± 15.7 a	328.5 ± 13.4 d	649.0 ± 7.4 b
CO_2 (first peak)	97.83 ± 3.34 b	350.0 ± 10.28 a	84.56 ± 3.26 b	328.6 ± 12.59 a

Values represent means ± SEM. Different letters indicate a significant difference between treatments ($n = 3$, $p < 0.05$).

different treatments (Figure 2). After the incubation (day 23), no significant differences were observed in the AOA, *nirS* and *nosZ* gene abundance between the treatments with the sheep urine application and without urine application (Figure 2A, D and E). The sheep urine application increased the soil AOB and *nirK* gene copies at the end of the incubation (Figure 2B, C). The AOB gene copies in the Bh + U treatment (7.7×10^6 copies g^{-1} soil) were significantly higher than that in the Br + U treatment (4.7×10^6 copies g^{-1} soil). The *nirK* gene copies in the Br + W (2.1×10^4 copies g^{-1} soil) were significantly lower than other treatments, but no significant differences were observed in the *nirK* gene copies between the Bh + W, Bh + U and Br + U treatments (3.3×10^4, 5.0×10^4, 3.7×10^4 copies g^{-1} soil, respectively).

4 | DISCUSSION

4.1 | Effect of Bh and Br on soil NH_4^+-N and NO_3^--N concentrations

The decrease of NH_4^+ and increase of NO_3^- in the treatments without sheep urine application was caused by the nitrification of residual soil NH_4^+. In the treatments with sheep urine application, the slight increase of NH_4^+ and marked increase in NO_3^- (over 200 mg N $kg\ soil^{-1}$) were caused by the hydrolysis of urea and further nitrification of the NH_4^+ from the urine-N applied (Byrnes et al., 2017). It was expected that soil with Bh retained significantly higher NH_4^+ and lower NO_3^- concentrations than soil with Br after the incubation, due to the biological NIs released from its (Bh) root to suppress the transformation of NH_4^+ to NO_3^- (Gopalakrishnan et al., 2009; Nuñez et al., 2018; Subbarao, Rondon et al., 2007). However, no significant differences were observed in the soil NH_4^+ and lower NO_3^- concentrations between the Bh and Br treatments in this study (Table 1).

Previous studies reported that soil applied with different amount of root exudates or compounds (which have been identified as biological NIs) from Bh retained higher soil NH_4^+ and lower NO_3^- concentrations compared with the bare soil treatments (Nuñez et al. 2018; Subbarao et al. 2006, 2008). Ma et al. (2021) found that soil applied with different concentrations of biological NIs (linoleic acid and linolenic acid) only decreased soil NO_3^- concentration but did not affect the soil NH_4^+ concentration due to the nitrification inhibition and/or N immobilisation. As for the effects of different *Brachiaria* species on soil nitrification, Castoldi et al. (2013) suggested that the levels of NH_4^+ and NO_3^- determined in the soil were similar among the *Brachiaria* species. This is consistent with the results in this study and also supported by the study by Castoldi et al. (2017), in which no significant differences were observed in the soil NH_4^+ and NO_3^- concentrations between *Brachiaria* species. Because of the need to retain air-tight seals throughout the incubation for the measurement of soil derived N_2 emissions, it was impossible to collect soil samples during the incubation period. A greater number of time points to explore the dynamics of soil NH_4^+ and NO_3^- during the incubation, would have helped to explain the effects of Bh and Br on the transformation of soil NH_4^+ to NO_3^-. Previous studies reported that the rates of nitrification inhibition increased with increasing concentrations of the biological NIs (Gopalakrishnan et al. 2009; Ma et al. 2021; Sun et al. 2016). The low stability of biological NIs released from Bh may be also one reason for the unexcepted results in this study. Ma et al. (2021) confirmed that biological NIs (linoleic acid and linolenic acid) identified from the shoot tissue of Bh were much more rapidly mineralised than synthetic NIs (such as DCD, less than 5% of mineralisation rate even after 40 days incubation) in a highly nitrifying soil, reaching 40% in about 10 days incubation.

4.2 | Effect of Bh and Br on soil N-gas and CO_2 emissions

N_2O and NO are known products of both nitrification and denitrification processes, which dominate under different optimal soil environment conditions such as soil moisture (Loick et al., 2016; Wu et al., 2017), pH (Robinson et al., 2014), temperature (Lai et al., 2019), O_2 availability (Senbayram et al., 2019; Zhu et al., 2013) and C availability (Miller et al., 2008; O'Neill et al., 2020). At the beginning of the incubation experiment, the initial soil water content was set as 65% WFPS which would have favoured nitrification of the NH_4^+ from the hydrolysed urea in the urine treatments causing the initial observed N_2O and NO emission peaks (first smaller peak). It is also supported by the study of Loick et al. (2021), in which nitrification was contributing the most to N_2O emissions at 70% WFPS. In addition, the initial CO_2 peak coincided with those of N_2O and NO, as a result of the amendment application, and provides evidence of aerobic respiration (Lee et al., 2011). The duration of this peak is similar to the first N_2O and NO peaks.

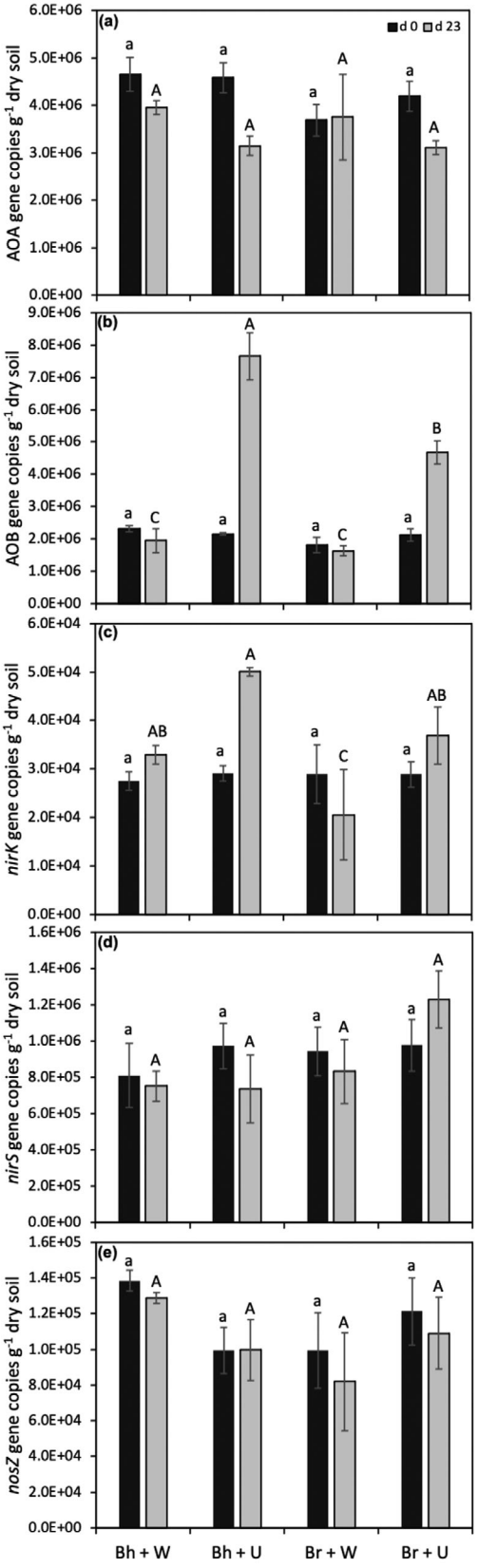

FIGURE 2 Average AOA (panel a), AOB (panel b), *nirK* (panel c), *nirS* (panel d) and *nosZ* (panel e) gene abundance at day 0 and day 23. Error bars represent standard error of the mean ($n = 3$). Different letters indicate significant differences between treatments at day 0 (lower case) and day 23 (upper case), respectively ($p < 0.05$)

Soil grown with Bh is assumed to have lower cumulative N_2O and NO emissions than that with Br due to the high BNI capacity in Bh (Gopalakrishnan et al., 2007; Subbarao et al., 2008). In this study, the cumulative N_2O in the Bh + U treatment during the first peak was significantly lower than that in the Br + U treatment, which may be due to the nitrification inhibition caused by the biological NIs released from the Bh as previously reported (Meena et al., 2014; Subbarao et al., 2006; Subbarao, Rondon et al. 2007). In addition, N_2O emissions factors (EFs) from sheep urine in the soil grown with Bh and Br were 0.41% and 0.43%, respectively, which is consistent with the literature review conducted by López-Aizpún et al. (2020) (with mean value of 0.39%, range from 0.04% to 1.80%). However, there was no significant difference in the cumulative N_2O and NO emissions during the whole soil incubation between the Bh + U treatment and Br + U treatment. It is possible that a reason for the short-lived effect of the Bh may have been the death of the grasses in the DENIS (there were no lights present in the incubation vessels). The residual biological NIs produced by the living plants prior to the incubation may have inhibited nitrification temporarily, but may not have remained effective after the death of the grasses.

4.3 | Effect of Bh and Br on Nitrifiers and denitrifiers

Synthetic NIs, such as DCD and DMPP, have been confirmed to inhibit the AOA and/or AOB genes copies, which play an important role in controlling the nitrification rates and dominate at different conditions (Chen et al., 2015; Li et al., 2019; Shi et al., 2016). NIs have also been shown to inhibit denitrifying microbes, *nirS* and/or *nirK* and/or *nosZ* and/or *narG* (Li et al., 2019; Shi et al., 2017; Zhou et al., 2018). The biological NI, 1,9-decanediol (identified from rice), has also been shown to suppress the nitrification through impeding both AOA and AOB, when applied at high concentrations (≥ 5 00 mg kg^{-1} soil) (Lu et al., 2019). A study conducted by Gopalakrishnan et al. (2009) also suggested that biological NIs released by the roots of Bh inhibited nitrifying bacteria, but did not negatively affect other major soil microorganisms. In this study, the controls (Bh + W and Br + W), did not influence the AOA, *nirS* and *nosZ* gene copies, but soil with Bh (with high BNI capacity) with sheep urine application significantly increased the AOB gene copies (responsible for the oxidation of NH_4^+) compared with Br (Figure 2). The AOA and AOB gene copies were not lower in the Bh treatments than Br treatments as excepted, which may be because biological NIs inhibit nitrification rates by reducing the cell-specific activity of AOA and/or AOB, rather than affecting ammonia oxidiser populations, as well as non-target soil microorganisms or functions (Kong et al., 2016).

In order to retain air-tight seals throughout the incubation for the measurement of soil derived N_2 emissions, soil samples were not collected during the incubation period. A greater number of time points to explore the dynamics of soil NH_4^+ and NO_3^-, as well as gene copies data during the incubation, or specific stable isotope approaches (such as ^{15}N labelling) would have helped to confirm the sources of gaseous N from soil grown with these two grasses, and nitrification inhibition

mechanism of Bh. Gopalakrishnan et al. (2009) suggested that BNI by roots of Bh varies with soil type. In addition, soil moisture content is an important factor related to the release of N-gas emissions (Loick et al. 2016; Wu et al. 2017). The effects of Bh on soil nitrification and GHG emissions under different soil moisture levels and soil types could be explored in the future studies.

5 | CONCLUSION

In this highly nitrifying soil, N_2O emissions dominated rather than NO emissions, from the soil sown with Bh and Br after the sheep urine application. Bh inhibited N_2O emissions during the first peak compared with Br, however, no significant differences were observed in the cumulative N_2O and NO emissions between the Bh + U and Br + U treatments over the entire 23 days incubation period. And there were also no significant differences in the soil NH_4^+ and NO_3^- concentrations between the Bh and Br treatments. We conclude that the residual biological NIs may inhibit the nitrification temporarily, but not last long enough in a highly nitrifying soil.

ACKNOWLEDGEMENTS

We would like to express sincere thanks to the technicians in Rothamsted research (North Wyke) for technical support during the study. This study is funded by the *Bangor-CSC scholarship (Bangor University and China Scholarship Council)*. Rothamsted is supported by the *BBSRC* (*BBS/E/C/000I0310* and *BBS/E/C/000I0320*).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Ma, Y., Charteris, A. F., Loick, N., Cardenas, L. M., Sha, Z., López-Aizpún, M., Chen, Q., Jones, D. L., & Chadwick, D. R. (2021). The short-lived inhibitory effect of *Brachiaria humidicola* on nitrous oxide emissions following sheep urine application in a highly nitrifying soil. *Journal of Plant Nutrition and Soil Science*, *184*, 723–732. https://doi.org/10.1002/jpln.202000501